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Award Number: W81XWH-07-01-0264

TITLE: Function of Klotho and its MicroRNA in Prostate Cancer and Aging

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REPORT DATE: October 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE 1 Oct 2008		2. REPORT TYPE Annual		3. DATES COVERED 15 Mar 2007 – 14 Sep 2008	
4. TITLE AND SUBTITLE  Function of Klotho and is MicroRNA in Prostate Cancer and Aging				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-07-1-0264	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S)  Shao-Yao Ying, Ph.D. Donald Chang  E-Mail: <a href="mailto:sying@usc.edu">sying@usc.edu</a>				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)  University of Southern California Los Angeles, CA 90033				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT We have observed the expression of CD164, IGFR, and Klotho proteins in human prostate cancer tissue microarrays as determined by immunohistochemistry. A positive correlation between CD164, IGFR and stages of prostate cancer was observed whereas a negative correlation between Klotho and stages was detected. However, the expression of Klotho in terms of the age of patients was not conclusive due to a small number of older (>65 years) patients. Previously, we have identified an intronic microRNA (miRNA) from the Klotho gene; one of the target genes of this miRNA is CD164, therefore, termed mir-CD164. We have conducted in situ hybridization of the mir-CD164 in non-cancer prostate and prostate cancer tissues. Staining was completely absent in prostate cancer samples whereas all of non-cancer tissues investigated showed strong staining for this miRNA, suggesting that human prostate cancer cells frequently have loss of the expression of mir-CD164. We have demonstrated a reverse correlation between the identified intronic miRNA of Klotho gene, mir-CD164, and prostate cancer. Mir-CD164-expressing vectors have been designed and their effect on CD-164 expression in prostate cancer cell lines is in progress.					
15. SUBJECT TERMS Progression of prostate cancer, Klotho, IGFR, CD-164, mir-CD164, microRNA and aging					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	7	19b. TELEPHONE NUMBER (include area code)

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## **INTRODUCTION**

The subject of this Exploration – Hypothesis Development grant is to test whether the novel intronic miRNAs identified in the first intron of Klotho gene plays a central role in prostate cancer during aging and to correlate the Klotho, IGFR, CD164 protein levels with the progression of prostate cancer and the age of prostate cancer patients. The methods used are immunohistochemistry and miRNA in situ hybridization methods in human prostate cancer microarrays. The purpose of this grant is to develop the concept by collecting evidence that there are correlation between the expression of these proteins and stages of prostate cancer and aging. By the same token, our purpose is also to establish the relationship of the expression of a miRNA derived from the intron of Klotho, mir-CD164 and the progression of prostate cancer. If this correlation can be established, a functional assay for mir-CD164 on the expression of CD-164 protein is examined. The scope of the research may explain some of the increasing aging-related incidence of prostate cancer and potentially has profound applicability on prevention and treatment of prostate cancer, using mir-CD164 in the silence of key genes associated with the progression of prostate cancer.

## **BODY**

In the Statement of Work, we stated that we would correlate the Klotho, IGFR, and CD164 gene and protein expression with the progression of prostate cancer and the age of patients, using human tissue arrays with different stages of prostate cancer, which will be completed before months 1-18. These tissue arrays are exempted and include multiple normal, BPH, and adenocarcinoma stages 1 to 4 corresponding to the Gleason scores 3-10. In addition, we will correlate the measurement with the condition of prostate cancer and the age of patients.

We have determined the immunoreactivity of CD164, and IGF-receptor (IGFR), and Klotho, proteins in human prostate cancer microarrays. For this series of experiments, we have acquired tissue arrays from Imgenex, San Diego, CA and Nxgen Biosciences, San Diego, CA, and these tissue arrays are exempted. The arrays include a total of 136 patients, staging from healthy, benign prostatic hyperplasia, early adenocarcinoma 1, malignant adenocarcinoma 2, invasive adenocarcinoma 3 and metastatic adenocarcinoma 4. We used a protocol previously established in the PI's laboratory with minor modifications for each specific immunohistochemistry. The sample slides were dewaxed with xylene and the primary antibodies (anti-Klotho polyclonal antibody, anti-CD164, goat polyclonal, and anti-IGFR antibody obtained from Santa Cruz Biotech, EMD Biosciences and Upstate Biomedicals, respectively), using immunohistochemical staining kits were obtained from Imgenex.

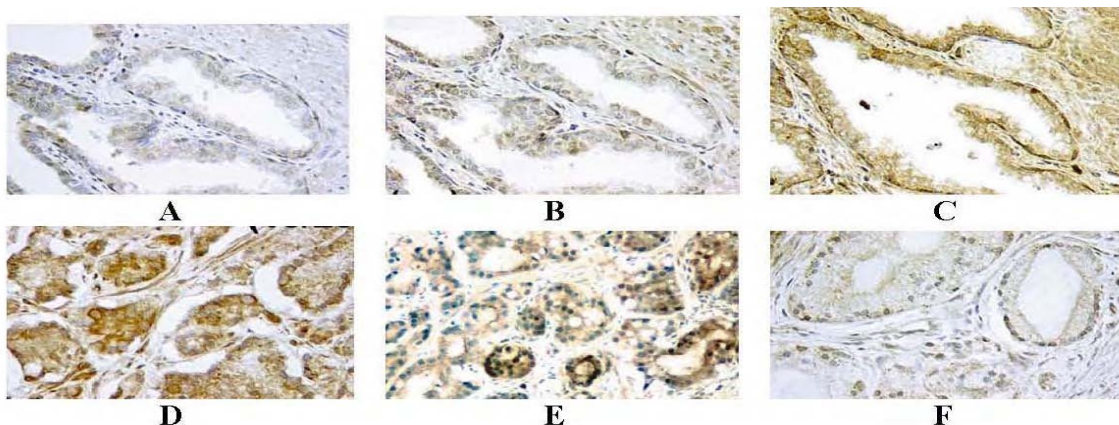


Figure 1. Examples of immunohistochemical detection of CD164 (A & D), IGFR (B & E), and Klotho (C & F) protein expression (magnification x400). A, B, and C are non-cancer prostate tissue and D, E, and F are stage 4 prostate cancer tissue (Gleason score 8-10).

Figure 1 shows examples of immunohistochemical detection of CD164, IGFR, and Klotho protein expression (magnification x400), indicating that expression of these proteins was observed in the epithelial cells of prostate cancer. Table 1 shows the correlation of the stages of prostate cancer and the immunoreactive expression of CD164, IGFR, and Klotho proteins, suggesting a positive correlation between CD164, IGFR and stages of prostate cancer whereas a negative correlation exists between Klotho and stages of prostate cancer. However, due to the small number of patients over 65 years of age, there appears a correlation between these protein expression and different age groups, it is not conclusive.

Table 1. Relationship of CD164, IGFR, and Klotho protein expression to prostate cancer stages

<b>Tissues</b>	<b>CD164 expression</b>	<b>IGFR expression</b>	<b>Klotho expression</b>
Non-cancer tissue (positive/total samples)	1/5 (20%)	3/6 (50%)	5/6 (83%)
Gleason score 3-4 (positive/total samples)	6/14 (43%)	6/13 (44%)	8/10 (80%)
Gleason score 5-7 (positive/total samples)	15/23 (65%)	20/22 (86%)	4/9 (44%)
Gleason score 8-10 (positive/total samples)	8/10 (80%)	12/12 (100%)	3/15 (20%)
Age (>65 years of age/all ages)	10/56	8/53	6/30

These results suggest that we have correlated the Klotho, IGFR, and CD164 gene expression with the progression of prostate cancer, but the correlation between the age of patients and the expression of these proteins was not clear due to the small number of samples investigated. In addition, our findings suggest that CD164 may participate in the progression of prostate cancer cells and provide further evidence that these proteins may be involved in prostate tumor progression and metastasis.

In the Statement of Work, we stated that we would identify the expression of a miRNA recently identified from the intron of Klotho gene in the above-mentioned tissue arrays, using miRNA in situ hybridization. We have measured miRNA-CD164 by *in situ* hybridization (ISH) expressions in human prostate cancer tissue arrays, using the *in situ* hybridization technique described by Kidner and Timmermans with minor modifications. FISH kits were purchased from Ambion, Inc. We used synthetic locked nucleic acid (LNA) probes (Sigma-Genosys) directed against the mature mir-CD164 sequences. The LNA-modified DNA oligonucleotide was a high-affinity RNA analog with a bicyclic furanose unit locked in an RNA-mimicking sugar conformation, which provided strong hybridization affinity toward complementary single-stranded RNA molecules. In a series of experiments, tissue arrays were dewaxed in xylene, rehydrated through an ethanol series (100%, 95%, 90%, 80%, 70%, 50%, 30%) and postfixed in 4% paraformaldehyde for 30 min. Then, the arrays were digested with proteinase K (10 µg/mL; Roche) for 5 min, refixed with 4% paraformaldehyde, and washed in Tris/glycine buffer. After that, the arrays were hybridized overnight at 60°C within coverslip chambers in *in situ* hybridization buffer (40% formamide, 5x SSC, 1x Denhardt's solution, 100 µg/mL salmon testis DNA, 100 µg/mL tRNA), containing 1 ng/µL of fluorescein-labeled LNA probes. After post-hybridization washes once with 5x SSC and once with 0.5x

SSC at 25°C for 1 h, positive results were observed under a 100x microscope with whole field scanning and recorded at 400x magnification (Nikon 80i microscopic quantitation system). As shown in Fig. 2, we have identified the intronic miRNA of Klotho (or mir-CD164) gene expression in prostatic epithelium (yellow arrows in A), which was greatly diminished in the advanced prostatic carcinomas with Gleason scores over 7 (yellow arrows in B). In addition, there was an inverse correlation between the loss of expression of mir-CD164 and the tumor grade. Of the 24 prostate cancer cases screened, staining was completely absent in 15 samples ( $P < 0.05$ ). In contrast, all thirteen samples of non-cancer tissues investigated in this study showed strong staining for this miRNA. These results, taken together, indicate that human prostate cancer cells frequently have loss of expression of mir-CD164. Furthermore, these observations provide a potential mechanism for prostate cancer cells to be modulated by miRNA derived from the intron of Klotho gene. Thus, we have demonstrated a reverse correlation with the identified intronic miRNA of Klotho gene, mir-CD164, and the advance of prostate cancer.

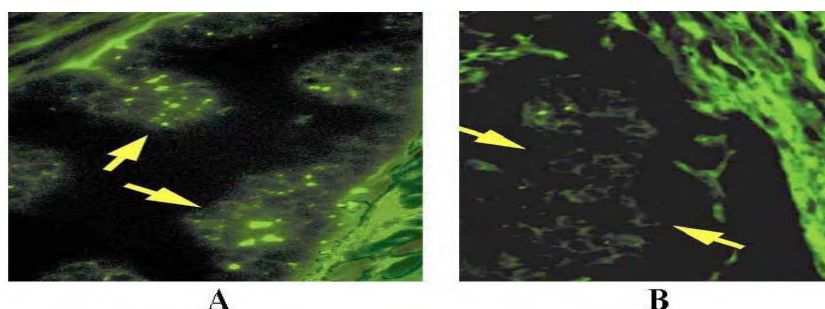


Fig. 2. Mir-CD164 in prostate tumors by in situ hybridization. Shown are representative examples of cytoplasmic expression of mir-CD164 in the prostate cancer epithelium. The fluorescent staining shows the presence of mir-CD164 in non-cancer tissues (A) whereas most tumors showed weak or no staining for mir-CD164 (B).

In the Statement of Work, we stated that we would determine the function of Klotho intronic miRNA, particularly on CD164 and IGFR in LNCaP, PC3, and DU145 cells, using man-made Klotho intronic miRNA technology. We have recently designed a man-made Klotho intronic miRNA according to the sequence identified and a retroviral vector delivery system based on a *cytomegalovirus* (CMV) promoter-driven *SpRNAi-RGFP* transgene for steady expression of the mir-CD164 in prostate cancer cells. We will test the function of this vector in prostate cancer cells, including LNCaP, PC3 and DU-145 cells. Then, the expression of CD-164 and IGFR proteins will be determined by Western blot analysis. We expect that the expression of CD-164 will be silenced by the expression of mir-CD164 in prostate cancer cells. Subsequently, we will determine the effect of mir-CD164 on the rate of cell proliferation, apoptosis, and cell invasion. Based on the observations described in Tasks 1 & 2, we hypothesized that the miRNA identified from the intron of Klotho gene may play an important role in cell proliferation and the expression of CD164 in prostate cancer cell lines. For this reason, a no-cost extension is request so that the functional studies of the mir-CD164 will be conducted.

### **KEY RESEARCH ACCOMPLISHMENTS**

- A positive correlation between CD164, IGFR and the stages of prostate cancer was established
- A negative correlation between Klotho and the stages of prostate cancer was established
- The correlation between Klotho and the age of patients was not established
- Mir-CD164 was positively expressed in prostate cancer tissues but not in non-cancer tissues

## **REPORTABLE OUTCOMES**

None

## **CONCLUSION**

The research has tested a novel concept that Klotho protein, an anti-aging molecule which has been shown to be associated with IGF-1 pathway, and an intronic miRNA derived from the Klotho gene have a correlation with the stages of prostate cancer as determined by immunohistochemistry and miRNA in situ hybridization, respectively. It is critical to note that the failure to demonstrate the correlation between Klotho and the age of patients does not mean that Klotho has no role in the normal aging process. Our findings also suggest that Kloth and/or mir-CD164 have the potential to play an important role in modulating function of the prostate gland. If mir-CD164 vectors are developed and found to be effective in prostate cancer cell lines in terms of inhibition of CD164 protein expression and cell growth, a new approach using miRNAs, to treat prostate cancer may open a new avenue in the fight against this disease. To substantiate this concept, we are anxious to conduct experiments that prostate cancer cells are treated with man-made mir-CD164 and to determine its effect on the expression of CD-164 protein and cell proliferation, apoptosis, and cell invasion.

## **REFERENCES**

None

## **APPENDICES**

None